Commercial yeast strains do not disseminate significantly in winery ecosystems of wine producing regions in France and Portugal

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Abstract

In order to obtain a better understanding of potential environmental risks associated to the use of genetically engineered winery yeast strains, a large-scale study was realized to assess their fate in the natural environment in different geographical localizations, using non-modified commercially available yeast strains as a model. The present study aims to evaluate the industrial starter yeasts' ability to survive and spread in nature, and become part of the natural microflora of musts.

In 6 different vineyards (3 in Portugal and 3 in France) that used the same industrial yeast strain(s) continuously in the last 5 years, sampling sites were chosen and grapes were collected in a pre- and post-harvest campaign. Towards the end of the spontaneous fermentations, the composition of the yeast flora was determined by different typing methods (PCR-amplification of ∂-sequences, pulse field electrophoresis, RFLP of mitochondrial DNA, and microsatellite typing).

The overall duration of these studies is 3 years, and the results obtained for the first two years showed that in Portugal, about 27% of the 930 isolates collected in 2001 and 2002 had identical typing patterns to the winery's starter yeast, but those isolates were derived from sites in proximity to the winery (20 – 40 m), close to sites with water runoff coming from the wineries. In France, where the samples were collected in a distance of 100 to 1000 m from the winery, only 2% of 1710 isolates collected in 2001 and 2002 revealed identical genetic patterns to the used industrial yeasts.

In global terms the obtained results showed that the dissemination of commercial strains in the ecosystem of a vineyard occurs in a limited space and time frame.

Introduction

Nowadays, about 50% of the European wine production is based on the use of active dried wine yeast. These strains were selected due to their good fermentation performance and to their capacity to produce a wine with desirable organoleptical characteristics. From an ecological point of view, they are non-indigenous, mostly *Saccharomyces cerevisiae* strains that are used without any containment and are annually introduced in large amounts, together with liquid and solid wine-making residues, in the ecosystem of the vineyard around the winery. The behaviour of these yeasts in natural habitats in different geographical regions is totally unknown as well as their potential impact on the natural microflora.

Only very few data are available that could contribute to the evaluation of the importance of starter yeast's dissemination and permanence in the vineyard (Frezier, and Dubourdieu, 1992; Vezinhet *et al.* 1992; Guillamón *et al*., 1996). Recently, a large-scale biogeographical study in South African vineyards was carried out during 4 years. In five areas situated in the Coastal Region vineyards of the Western Cape 13 samples were collected and commercial yeasts were recovered from 3 samples (van der Westhuizen *et al*., 2000a and 2000b).

In order to obtain a better understanding of potential environmental risks associated to the use of genetically engineered winery yeast strains, a large-scale study was established to assess their fate in the natural environment in different geographical localizations, using commercially available yeast strains as a model. The present study aims to evaluate the industrial starter yeasts' ability to survive and spread in nature, becoming part of the must microflora.

Materials and Methods

The sampling plan included 36 sites in 6 vineyards, being 3 located in the South of France (Languedoc) and 3 in the North of Portugal (Região Demarcada dos Vinhos Verdes), as shown in figure 1. In Portugal, winery 1 is located in the South (Baião), winery 2 (Ponte de Lima) in the Centre and Winery 3 (Monção) in the North, the distance between each other is above 30 km. In France, the wineries are located in the Region of Languedoc, around the Mediterranean city of Montpellier, and the distance from each vineyard to another is comprised between 30 and 80 km. The overall duration of these studies is 3 years (2001-2003), here we report data of two years work.

The wineries selected used consecutively one or more commercial yeast strains (table 1) in the last 5 years. The 3 Portuguese wineries used mainly Zymaflore VL1 (Lallemand), a strain originally selected in France, while the 3 French wineries used predominantly K1M ICV-INRA.

In each vineyard, six sampling points were defined according to the local conditions (size and orientation of the vineyard, predominating wind direction), and the distance between winery and the sampling sites varied between 20 to 1000 m, as shown in figure 1. In order to evaluate whether commercial yeast strains can survive outside the winery from one year to another, a first sampling campaign was performed (pre-harvest campaign), when the winery had not yet started the wine production. In a second post-harvest sampling campaign, the grapes were collected after the onset of the wine production, in order to evaluate the immediate commercial yeast dissemination from the winery. With the present experimental design, 72 grape samples were collected each year.

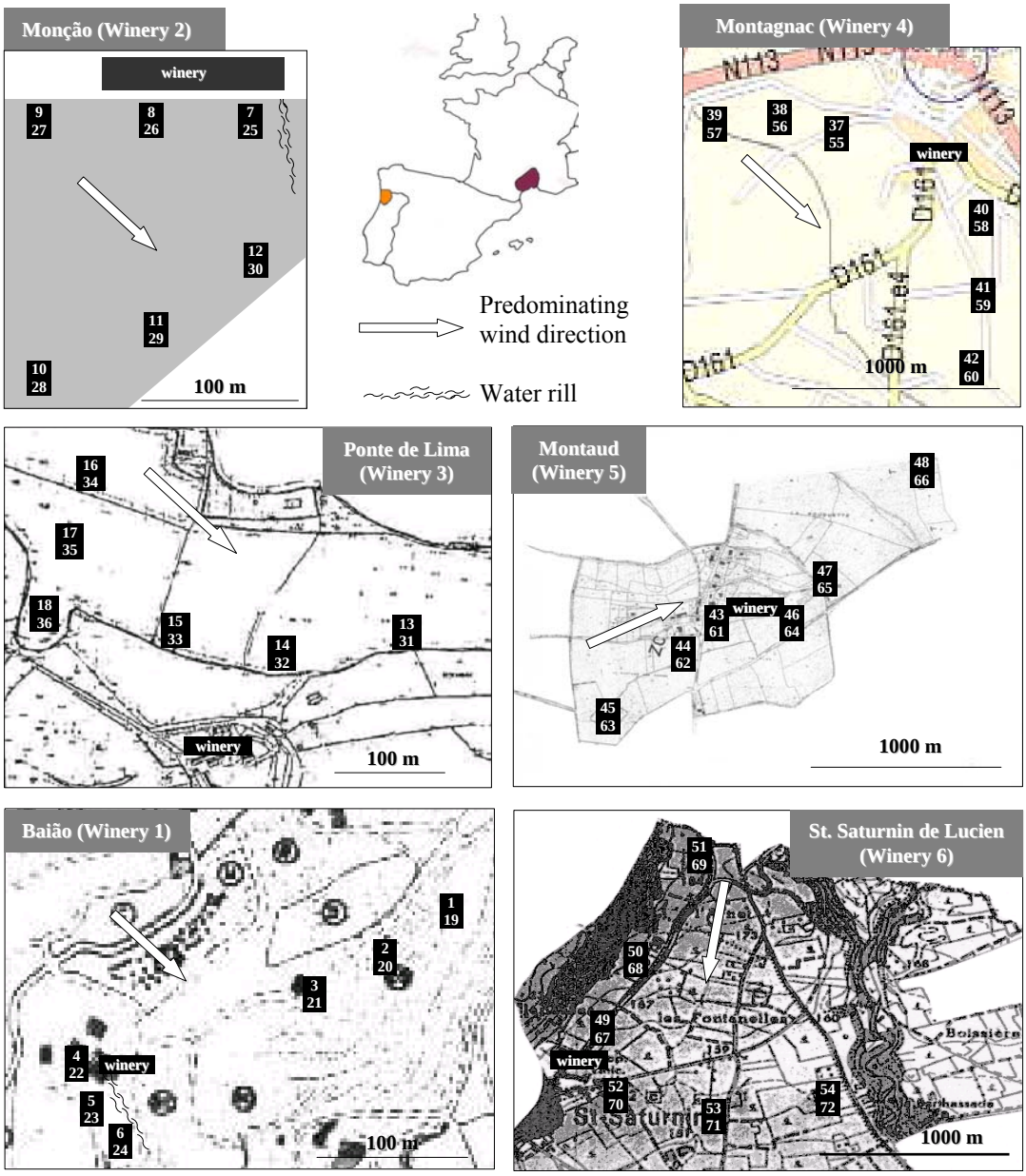

Figure 1

Geographic localization of the Vinho Verde and Laguedoc wine regions and indication of the sampling points in the six vineyards. In each sampling site, the first number refers to the pre-harvest sample and the second to the post-harvest sample, respectively.

Table 1

Commercial yeast strains that were used in each winery in the last years. The underlined strains have been used consecutively for the last 5 years.

Winery 1	Winery 2	Winery 3	Winery 4	Winery 5	Winery 6
ZymafloreVL1	Zymaflore VL1	Zymaflore VL1	K1M ICV-INRA	K1M ICV-INRA	K1M ICV-INRA
Zymaflore F10	Lalvin EC 1118	Lalvin EC 1118	Zymaflore VL3	ICV D254	ICV D254
ZymafloreVL3	Levuline BRG		Maurivin PDM	ICV D80	ICV D47
Zymaflore F15	Fermichamp ®		ICV D254	Uvaline BL	Enolevure K34
Lalvin QA23			ICV D47	Lalvin BM45	Lalvin QA23
Lalvin CY 3079			Lalvin EC 1118	Maurivin AWRI2	
ICV D47			Uvaline arôme		
ICV D254			Chardonnay MV		
Uvaferm L2056			Anchor Vin 13		
Uvaferm BDX					
Uvaferm ALB					
Uvaferm 228					
Uvaferm CS2					

From each sampling point, approximately 2 kg of grapes were asseptically collected, and the extracted grape juice was fermented in small volumes (200 – 500 ml), with mechanical agitation at 20ºC. Daily weight determinations allowed the monitoring of the fermentation progress. The yeast flora was analysed when the must weight was reduced by 70 g/l, corresponding to the consumption of about 2/3 of the sugar content. Must samples were diluted and spread on plates with YPD medium (yeast extract, 1% w/v, peptone, 1% w/v, glucose 2% w/v), and after 2 days of incubation 30 randomly selected colonies were collected from each spontaneous fermentation and subjected to molecular identification.

Results and discussion

In order to evaluate the best method that allowed a fast and precise high-throughput screening, different approaches have been adopted. A first screening was performed using YNB medium (Difco) with lysine as the only nitrogen source. All isolates that were not able to grow on this medium, but grew on the control medium YNB with ammonium sulfate were considered as belonging to the genus *Saccharomyces*, and were further analysed by chromosomal pulse field gel electrophoresis.

As molecular screening, the interdelta sequence amplification patterns (Ness *et al.*, 1993) of the isolates were compared to the ones of commercial strains. This method has a reduced discriminatory power, and all isolates having identical patterns to those of the commercial yeasts were further analysed by mitochondrial DNA restriction analysis, using *Hinf* I (López *et al*., 2001). A very good correspondence between the discriminatory power of electrophoretic karyotyping (Blondin *et al*., 1988) and mitochondrial restriction analysis was found. The isolates with identical mitochondrial DNA restriction patterns to those of the used commercial yeast strains, were further analysed by microsatellite typing (Gallego *et al*., 1998;

González Techera *et al.,* 2001; Hennequin *et al*., 2001), using 6 previously described *loci* (Pérez *et al*., 2001). The results obtained by mitochondrial restriction analysis were confirmed and in addition, indicated also some microevolutionary changes in the genome of the analysed isolates. Studies are now underway in order to evaluate the probable time frame of the permanence of those strains in nature based on the frequency of small size changes in the microsatellites.

The results obtained for the six wineries during the pre- and post-harvest campaign are summarized in table 2.

In winery 1 (Baião), three sampling points are in close proximity to the winery (20 – 40 m) (figure 1), being the other 3 points located in a distance of 150-250 m. All isolates from this winery belong to the genus *Saccharomyces sp*. During the last 5 years the strains Zymaflore VL1 and F10 have been continuously used, besides other yeast strains that were used sporadically (table 1). Both strains were found in the sites with closer proximity to the winery, predominantly in fermentations from grapes collected after the harvest, where they contributed to 50 – 100 % of the yeast flora (samples 21,22,23 and 24), indicating that these strains may occur in the vineyard due to the immediate release with the onset of the wine production (table 2). Zymaflore F15 was found predominantly in samples collected before the beginning of the wine production in 2001 (samples 4,5 and 6), and its presence could indicate a higher capacity to survive in the vineyard than Zymaflore VL1 and F10. Additionally, F15 was also found in site 3/21, located about 100 m from the winery. The strain ICV D254 was abundant in two post-harvest samples from the sites close to the winery. In 2002, due to heavy rain falls and a very bad sanitation condition of the grapes, many samples could not be collected. No commercial yeast strains have been found in the sole spontaneous fermentation. The existence of a small rill close to the winery, where water runoff from the winery flows, due to the inclination of the terrain, could be an important vector for the spreading of commercial yeasts in the vineyard. The presence of the strains Zymaflore F15, F10 and ICV D254 in more distant regions of the vineyard may be attributed to other factors like the transmission by wind or insects.

In winery 2, (Monção), the strain Zymaflore VL1 was detected in the post-harvest samples of the grapes collected in 2001 in sites that are 10 to 30 m away from the winery. Several isolates were found in both years with a genetic pattern identical to the strain Lalvin QA23. This yeast strain has been initially selected from the Vinho Verde Region, therefore it was not possible to decide whether these isolates correspond to the natural yeast flora or were disseminated from the winery. The fact that strains with the genetic pattern of QA23 were found in sites close to the winery favours the dissemination hypothesis.

Two isolates were found with the genetic pattern of strain ICV D254. It is noteworthy that also in this winery the samples where the highest amount of commercial yeast strains were detected, are located close to the rill that transports runoff water from the winery, emphasizing the importance of water as a vehicle for yeast strain dissemination. In all fermentations, the yeast flora contained only *Saccharomyces sp*. strains.

In winery 3, samples were collected in distances ranging from 150-400 m to the winery. None of the 270 *Saccharomyces sp*. strains isolated in 2001 and 2002 campaigns had the genetic patterns of the commercial yeast strains that were used since 5 years in this winery (Zymaflore VL1 and Lalvin EC 1118).

Table 2

Composition of the yeast flora isolated from all samples of the 6 wineries. 1: percentage of *Saccharomyces sp*. strains among the total of 30 isolates per fermentation; 2: number of genetic profiles in each fermentation; 3: percentage of isolates identical to commercial yeast strains genetic pattern

Winery	Sample	2001						2002					
		Pre-harvest			Post-harvest			Pre-harvest			Post-harvest		
		1	2	3	1	2	3	1	2	3	1	2	3
1	1/19	No fermentation			No fermentation			Not collected			Not collected		
	2/20				100	20	3						
	3/21				100	4	97						
	4/22	100	5	100	100	2	100	100	1	0			
	5/23	100	4	80	100	4	100	No fermentation					
	6/24	100	1	100	100	8	50						
2	7/25	100	2	0	100	5	87	No fermentation			100	4	0
	8/26	100	2	0	100	15	50				100	5	23
	9/27	No fermentation			100	8	0	Not collected			100	1	0
	10/28				100	18	0				No fermentation		
	11/29				100	18	0				100	9	40
	12/30				100	15	33				100	1	0
3	13/31	No fermentation			No fermentation			Not collected			100	18	0
	14/32				100	2	0				100	2	0
	15/33				100	8	0				100	10	0
	16/34				No fermentation						100	6	0
	17/35				100	1	0				100	9	0
	18/36				No fermentation						100	3	0
4	37/55	93	1	0	0	-	-	0	-	-	7	1	0
	38/56	No fermentation			100	1	0	0	-	-	0	-	-
	39/57	80	20	0	40	4	0	0	-	-	0	-	-
	40/58	67	5	0	57	10	0	0	-	-	0	-	-
	41/59	93	7	0	93	2	0	0	-	-	0	-	-
	42/60	0	-	-	46	12	0	0	-	-	0	-	-
5	43/61	No fermentation			100	2	0	0	-	-	0	-	-
	44/62				97	1	0	0	-	-	47	1	0
	45/63	33	1	33	3	1	0	0	-	-	0	-	-
	46/64	3	1	0	97	14	0	0	-	-	0	-	-
	47/65	17	1	17	0	-	-	0	-	-	73	1	0
	48/66	No fermentation			0	-	-	No fermentation			0	-	-
6	49/67	No fermentation			87	1	0	0	-	-	0	-	-
	50/68	0	-	-	No fermentation			No fermentation			0	-	-
	51/69	No fermentation						0	-	-	0	-	-
	52/70				100	1	0	0	-	-	0	-	-
	53/71				0	-	-	0	-	-	No fermentation		
	54/72				No fermentation			0	-	-	3	1	0

In general, in the wineries located in the Languedoc Region, samples have been collected in a greater distance from the winery than in Portugal. Wineries 4, 5 and 6 used the marked strain K1M ICV-INRA continuously for at least 5 years.

No commercial yeast strains have been detected in Montagnac (winery 4) during both sampling years. Interestingly, non-*Saccharomyces* species were involved in numerous fermentations. The 2002 fermentations were clearly dominated by non-*Saccharomyces* species, less than 1% of the isolates belonged to the genus *Saccharomyces*.

In Montaud (winery 5), the karyotype of 15 isolates was identical to the commercial strain ICV D254. They were found in the pre-harvest samples (45 and 47) collected in 2001, representing 17 and 33% of the flora, respectively. Both samples were collected from two opposed directions relative to the winery. These findings could indicate a previous dissemination, but this possibility can not be confirmed, since this commercial yeast strain has been selected from the region of Languedoc. Only 40% of the isolates collected in 2001 were *Saccharomyces sp*. yeasts, and in 2002 this number decreased to 11 %.

The sixth winery was located in Saint-Saturnin de Lucian. In both sampling years, none of the isolates had an identical genetic pattern with any of the commercial strains used for the last 5 years. Only 47 % of the isolates collected in 2001 were *Saccharomyces sp*., and this number was much lower in 2002, being just 1% of the yeast flora *Sachharomyces sp*.

Figure 2 shows the global data obtained in this study. A total of 1290 isolates have been collected from spontaneously fermenting grape must during 2001 (figure 2). From the fermentations performed with grapes collected before the beginning of the harvest, 85 of the 420 isolates (corresponding to 20 %) had an identical genetic profile to the commercial yeast strains that have been used in the 6 wineries. The same was verified for 161 of the 870 isolates (corresponding to 19 %) originating from post-harvest samples.

In the pre-harvest samples of winery 1, the yeast strains Zymaflore F15 was predominant in 2001, indicating that this yeast might have survived in the vine, contrarily to the strains Zymaflore VL1 and ICV D254 that were found only in the post-harvest samples. Zymaflore VL3, a strain used continuously for the last 5 years in the winery 1 could not be detected, a fact that evidences differences among starter yeasts concerning their capacity to survive in the vine. The presence of the strains QA23 in the Vinho Verde Region and D 254 in Languedoc due to dissemination can not be affirmed, as these strains were selected from the two regions in Portugal and France respectively.

Unfavourable weather conditions with heavy rain falls before the harvest contributed in 2002 in Portugal to a very bad sanitation state of the grapes, and not all grape samples could be collected. In France, rainfall was also registered during the harvest period. None of the 510 isolates collected in both wine regions before the harvest had a genetic pattern corresponding to the commercial strains, and only 19 isolates (2%) of the 840 post-harvest isolates corresponded to the commercial strain QA23.

Among the 930 isolates from Portugal, 210 different genetic patterns were found regarding the indigenous flora. In 2001 the strains with identical genetic patterns to commercial starters dominated the fermentations in samples 4, 6, 21, 22 and 23. Indigenous strains contributed 40-70 %, where 5 to 15 different genetic patterns could found (samples 24, 26 and 30 of 2001 and 26, 29 of 2002). In two cases, less than 10% of commercial strains were detected, the

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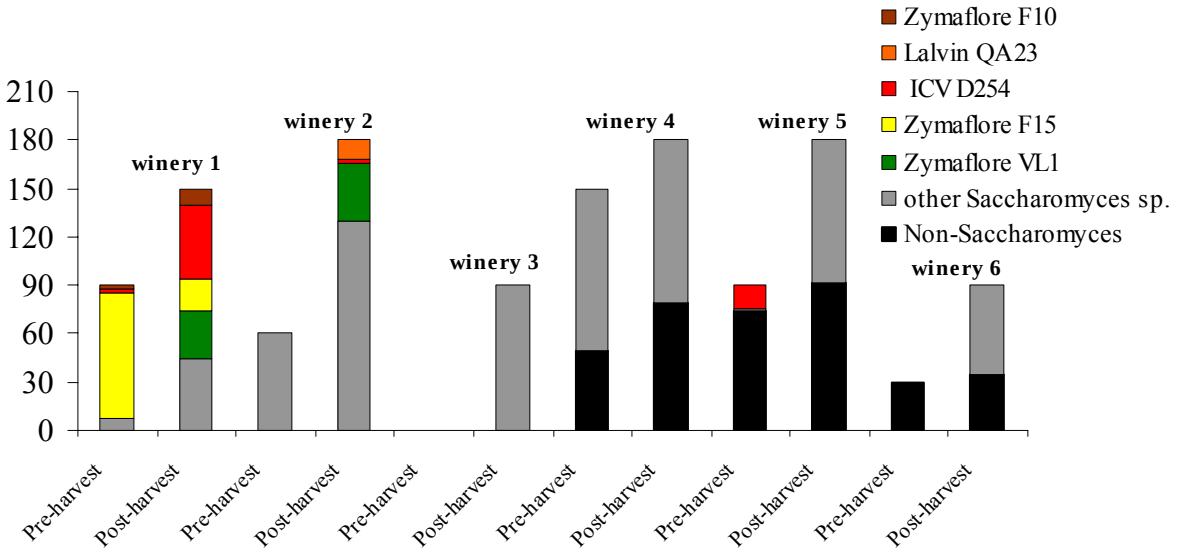

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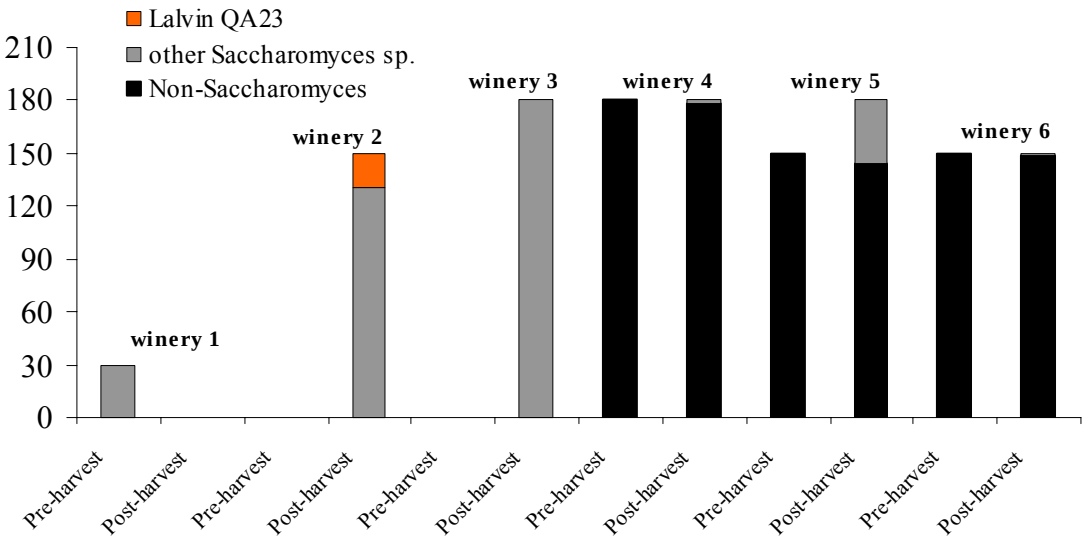

Figure 2

Global composition of the yeast populations isolated from the 6 wineries during the pre- and post-harvest sampling campaigns in 2001 (A) and 2002 (B).

indigenous yeast flora revealed a very high biodiversity (20 different strains in sample 20 and 18 different strains in sample 28 from 2001).

From the 1710 isolates collected in France during both years, only 438 (25.6 %) corresponded to the genus *Saccharomyces sp*. Among them, about 87 different genetic patterns have been found. In the two fermentations where the starter yeast ICV D254 has been detected (sample 45 and 47 of 2001), no other *Saccharomyces* strains were detected, being the accompanying indigenous flora non-*Saccharomyces* strains. The substantial increase of non-*Saccharomyces*

strains in 2002 could be attributed to distinct climatic conditions, as rainfalls occurred in the harvest period.

Concluding Remarks

Based on the results obtained from two sampling years, the dissemination of commercial yeast strains can be observed in sites that are close to the winery, mostly at a distance of 20 – 40 m, until a maximum distance of about 150 m. Samples collected at a distance above 150 m did not contain starter yeasts. In France, 2 samples collected at a distance of 300-500 m from the winery contained isolates with identical genetic patterns to a commercial strain that was initially isolated from the South of France. It is therefore not possible to determine whether these isolates made part of the indigenous flora, or their presence resulted from dissemination. The capability of commercial strains to survive in the wineries ecosystem from one year to another varied among the strains, several strains were not able to survive or dominate the natural flora of the vine from one year to another, and their dissemination should be considered as limited in space and time. Water runoff that flows in a rill from the winery to the vine due to the inclination of the terrain may be an important vector for the spreading of commercial yeast strains. As the fermentative microflora on the grape surface undergoes multiplication during fermentation, the determined percentage of commercial yeast does not reflect the natural situation in the vine, and it should be noted that the present results do not allow conclusions about the number of strains occurring on the surface of the grape, that may be in fact very low.

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